Involvement of Th17 cells and the effect of anti-IL-6 therapy in autoimmune uveitis

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Objectives. Human endogenous uveitis is one of the sight-threatening diseases associated with variety of systemic disorders, such as Behcet's disease and sarcoidosis. Recently, biosynthesized antibodies against inflammatory cytokines have been recognized to be useful to control the regional inflammation. In this study, we focused on the possibility of IL-6-based biological therapies for endogenous uveitis. We initially confirmed the significant increase of several inflammatory soluble factors including IL-6 in the vitreous fluids from refractory/chronic enogenous uveitis patients.

Methods. To investigate the role of IL-6 in the formation of refractory ocular inflammation, we used the mouse experimental autoimmune uveitis (EAU) model. Both IL-6 and IL-23 are required for the development of IL-17-producing helper T subset (Th17) from naïve CD4$^+$ T cells.

Results. In the EAU model, neither IL-6-deficient mice nor IL-23-deficient mice could induce Th17 cells and the EAU score was decreased in these mice in the entire time course. We also confirmed that systemic administration of anti-IL-6 receptor antibody ameliorates EAU by suppressing both systemic and regional Th17 responses.

Conclusions. IL-6 is responsible for causing ocular inflammation, and it is, at least partially, due to IL-6-dependent Th17 differentiation. IL-6 may be a target for therapy of refractory endogenous uveitis in humans.

KEY WORDS: Autoimmunity, Cytokine, Eye, IL-6 receptor, Th17.

Introduction

Endogenous uveitis, such as Behcet's disease, Vogt–Koyanagi–Harada disease, Sarcoidosis and juvenile idiopathic arthritis (JIA), is known to be one of the sight-threatening intraocular diseases. Complications, such as cystoid macular oedema and proliferative vitreoretinopathy, can cause permanent loss of vision. In many cases of uveitis, several immunosuppressive drugs (e.g. corticosteroids, colchicines, cyclosporins) are needed to control the inflammatory process in the eye. However, long-term administration of these drugs can cause severe side effects, such as osteoporosis, infertility, diabetes and infections.

Biologic agents include mAbs and recombinant forms of natural inhibitory molecules has been invented, and applied in several inflammatory chronic diseases including 'eye disease' [1, 2]. For instance, infliximab is a chimeric monoclonal antibody directed against the soluble and membrane-bound forms of TNF-α inhibitors: it has been used and reported to be effective in RA [3], SpAs [4], Crohn's disease [5] and psoriasis [6]. Several studies have demonstrated that infliximab is effective for the refractory ocular inflammation due to Behcet's disease [7–9]. Beside infliximab, etanercept, human soluble TNF-α receptor fusion protein and adalimumab, fully human monoclonal antibody to TNF-α, have been reported to be effective to refractory uveitis [10]. But anti-TNF-α agents were not perfect and can cause autoimmune diseases, principally cutaneous vasculitis, lupus-like syndrome [11]. We thus still need to seek other biologics that are more effective and have fewer side effects.

IL-6 is a pleiotropic inflammatory cytokine produced by T cells, monocytes, macrophages and synovial fibroblast and mediate various functions through its specific receptor (IL-6R). Several lines of evidence have shown that an excessive amount of IL-6 causes various inflammatory autoimmune diseases, such as RA and SLE, Castleman's disease, systemic-onset JIA and some other inflammatory diseases [12]. Perez *et al.* [13] reported that IL-6 was elevated in the vitreous body of patients with active intermediate and posterior uveitis. Due to the findings from the autoimmune models in IL-6 knockout mice including experimental autoimmune encephalomyelitis (EAE) and CIA, IL-6 has been shown to be essential for inducing inflammation [14]. The role of IL-6 in RA was well analysed, and tocilizumab, a recombinant humanized anti-IL-6 receptor antibody, has shown to be effective to several diseases in both experimental [15, 16] and clinical [17] settings.

From another point of view, IL-6 is essential for differentiation of Th17, a recently discovered IL-17-producing helper CD4$^+$ T-cell subset [18, 19]. Th17 constitutes a Th cell lineage distinct from Th1 and Th2 cells, and plays a crucial role in several autoimmune diseases by mediating tissue inflammation [20]. IL-17 stimulates IL-17R-expressing cells, such as epithelial, endothelial and fibroblastic stromal cells, and these cells in turn induce the secretion of IL-6, IL-8, PGE2, MCP-1 and G-CSF [21], causing chronic inflammation. Moreover, IL-23 has been reported to be responsible for Th17 expansion and critical for the development of autoimmune inflammatory diseases [22, 23]. Langrish *et al.* [22] demonstrated that lymph node cells cultured with IL-23, but not IL-12, produce vast amounts of IL-17, and then cause severe disease (EAE) by adoptive transfer. IL-23 is a member of the IL-12-related cytokine family and contains the p40 subunit, which is common to IL-12 and IL-23, and a unique p19 subunit [22]. Chen *et al.* [24] showed that administration of anti-IL-23p19 mAb ameliorated EAE, with reduced serum level of IL-17 as well as CNS expression of pro-inflammatory cytokine. Thus, it is now believed that Th17 development is dependent on the presence of both IL-23 and IL-6.

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Based on the above findings, we considered that IL-6 might be a therapeutic target to treat endogenous uveitis. To access the therapeutic potential of anti-IL-6 reagents to uveitis, we used the animal model of human uveitis, experimental autoimmune uveitis (EAU) [25, 26]. Several lines of evidence have shown that autoreactive Th1 cells mediate EAU [27, 28]; that its induction is correlated with the production of IFN-γ by T cells; and that inhibition of Th1 responses by anti-IL-12 treatment suppresses the disease [29]. Since Th17 cells have also been suggested to be involved in EAU [30, 31], we thus analysed the role of Th17 cells on EAU by using IL-6- and IL-23-deficient mice. We confirmed that EAU development was reduced in these mice. Therefore, we have tried systemic administration of recombinant anti-IL-6 receptor antibody and found that anti-IL-6 therapy ameliorates EAU and interferes with antigen-specific Th17 differentiation/ expansion.

Materials and methods

Vitreous and aqueous humor specimens

Human undiluted vitreous fluid (200–900 μl) was collected during pars plana vitrectomy, prior to intraocular fluid infusion. Vitreous samples were obtained from 35 eyes of chronic uveitis patients and 83 eyes of control patients. Vitrectomy against vitreous opacity and/or macular oedema due to refractory/chronic inflammation was performed on all enrolled uveitis patients. As a control, we selected either patients with idiopathic macular hole or epiretinal membrane, who were free from major pathogenic intraocular changes, such as ischaemia, inflammation and proliferative membranes. For another control, aqueous humor was collected from six acute uveitis patients (within 48 h from onset). Samples were immediately placed in sterile, 1.5-ml polypropylene tubes on ice, and stored at -70°C until assay. After diluting $10 \times$ with PBS, the concentration of cytokines and chemokines, and growth factors of vitreous specimens were measured using a microbead-based ELISA system (Human Cytokine Kits, BioSource International, Camarillo, CA, USA), according to the manufacturer's directions, with Luminex 100 (Luminex, Austin, TX, USA). The research followed the tenets of the Declaration of Helsinki and the Ethics Committee of Kyushu University approved the study. Informed consent was obtained from all enrolled patients.

Mice

IL-6-deficient, IL-23p19-deficient [32] in C57BL/6 background (maintained for more than 10 continual crosses) and wild-type (WT) C57BL/6 mice (SLC Japan, Shizuoka, Japan) were maintained in specific pathogen-free conditions at Kyushu University, Japan. All animals were treated humanely, and all experiments conformed with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

EAU induction and evaluation

WT C57BL/6, IL-6-deficient and IL-23p19-deficient mice were immunized with human interphotoreceptor retinoid-binding protein (IRBP) peptide 1-20 (GPTHLFQPSLVLDMAKVLLD), as described previously [33]. The mice were first immunized subcutaneously in one footpad and the base of tail with the peptide in 0.2 ml emulsion of CFA (1 : 1, vol/vol), supplemented with *Mycobacterium tuberculosis* strain H37RA to 2.5 mg/ml, and then inoculated intraperitoneally with 100 ng of pertussis toxin. Ophthalmic examinations were carried out following immunization. Tropicamide (0.5%) was applied to the eyes to induce mydriasis, and the fundus of the eye was examined using a Bonnoscope and Super Field NC Lens (Volk Optical, Mentor, OH, USA). EAU severity was assessed both clinically and histopathologically in a blind manner as described elsewhere [25, 34].

Treatment with an IL-6R-neutralizing antibody

Animals were treated with intraocular or intraperitoneal injection of a rat anti-mouse IL-6R monoclonal antibody MR16-1 (Chugai Pharmaceutical Co. Ltd, Tokyo, Japan), prepared as described previously [35], or a purified rat non-immune isotype control IgG (Sigma-Aldrich, St Louis, USA) from the day after immunization as described. MR16-1 has been described to bind to murine IL-6R and suppress IL-6-induced cellular responses [36]. MR16-1 was injected into mice with 1 μl per eye of 10 μg/ml intravitreously or 10 μg/g body weight intraperitoneally.

Vitreous cavity injection

MR16-1 (1 μl per eye of 10 μg/ml) was injected into the vitreous cavity (VC) using fine 32-ga. needles (cat. no. 0160832, Hamilton, Reno, NV, USA) and a 10-μl syringe (cat. no. 80330, Hamilton). The tip of the needles penetrated the sclera, choroid and retina, to reach the VC and a maximum volume of 2 μl per injection was introduced into the VC. We were confident that we were injecting the antibody into the VC, because the tip of the needle was carefully guided under the microscope through a flattened cornea covered by a glass microscope slide. After inoculation of 1 μl solution, elevated intraocular pressure completely sealed the retinal incision without bleeding and detachment.

Ocular fluid preparation and protein assay

Eyes were enucleated under deep anaesthesia, the conjunctival tissue was removed and the remaining eye tissues (cornea, iris, vitreous body, retina, choroids and sclera) were homogenized using a Biomasher (Nippi Inc., Tokyo, Japan). After centrifugation at $12\,000 \times g$ for 30 min, supernatants were collected, and the concentration of cytokines were measured using a microbead-based ELISA system (Mouse Cytokines Twenty-Plex antibody bead kit, Catalog #LMC0006, BioSource International,), according to the manufacturer's directions, with Luminex 100 (Luminex).

Cytokine ELISA and intracellular staining

The cervical, submandibular and inguinal lymph nodes (LNs) were removed from four or five mice on indicated days post-immunization. CD4$^+$-enriched cells were prepared using anti-CD4 Microbeads (130-1-049-201; Miltenyi Biotec, Gladbach, Germany) and an MS positive-selection column with a MiniMACS$^{\text{TM}}$ Separator (Miltenyi Biotec). The suspended CD4$^+$ cells (purified to ~95% by adherence to the column) were incubated with or without IRBP peptide (10 μg/ml) at a concentration of 2×10^5 cells/200 μl/well for 48 h at 37°C. Irradiated (20 Gy) spleen cells from WT mice were used as antigen-presenting cells (APCs) in the cultures at a concentration of 1×10^6 cells/200 μl/well. Supernatants were collected and cytokine concentrations were measured using ELISA development kits (eBiosciences) for detection of IL-17 and IFN-γ according to the manufacturer's directions. For anti-mouse allophycocyanin-Foxp3 (FJK-16s) (eBiosciences, San Diego, USA) intracellular staining, LN cells were labelled with anti-CD4 Abs, fixed and permeabilized, according to the manufacturer's protocol.

Statistical analysis

Unpaired Student's *t*-test for parametric data (cytokine production) and Mann–Whitney U-test for non-parametric data (EAU scores and ocular fluid protein assay) were used to analyse differences between groups of mice. $P < 0.05$ was considered to be statistically significant. In cytokine data, the correlationship with three factors (IL-6, IL-8 and MCP-1) in many individuals was examined by Spearman's test.

Results

Increased inflammatory cellular mediators in ocular fluid from patients with chronic endogenous uveitis

Endogenous uveitis has been considered to be intricately affected by several cellular mediators, such as cytokines, chemokines and growth factors [13]. We thus attempted to measure the concentrations of multiple cellular mediators using microbead-based multiplex ELISA system, and then analysed the relationship of each factor in causing ocular inflammation. As shown in Fig. 1, in vitreous samples from refractory/chronic uveitis, significant increases of IL-6, IL-8, MCP-1 were observed compared with control subjects from patients with idiopathic macular hole or epiretinal membrane (that were free from major pathogenic changes). Moreover, a strong correlation was observed between IL-6 vs IL-8 (Spearman's correlation $r = 0.6398$, $P < 0.0001$), but not IL-6 vs MCP-1 or IL-8 vs MCP-1 ($r = 0.2688$ and 0.2805; $P = 0.1184$ and 0.1026, respectively). We considered three elevated factors (especially IL-6) that are produced by bone marrow-derived immune cells, and being certainly tissue destructive, may also act for pathogenic T-cell differentiation in systemic circulation.

We would like to emphasize that vitrectomy against vitreous opacity and/or macular oedema due to 'refractory/chronic' inflammation was performed on all enrolled uveitis patients. Some major inflammatory cytokines including IL-2, IL-1β and IFN-γ
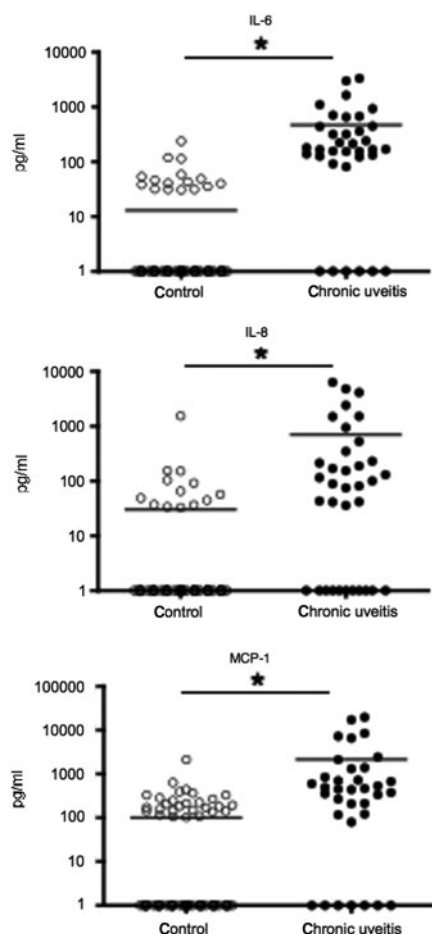

FIG. 1. Concentrations of IL-6, IL-8 and MCP-1 in the vitreous fluid. Vitreous samples were collected from patients with non-proliferative control diseases (epiretinal membrane and macular hole: $n = 83$) and chronic uveitis (Behcet's disease, sarcoidosis, Vogt–Koyanagi–Harada disease and unknown uveitis: $n = 35$). Concentrations of indicated cellular mediators were analysed. $^*P < 0.01$.

were under the detection level in 'all' vitreous samples. IFN-γ has been reported to be detected in the aqueous humor of 'acute' uveitis [37]. In accordance with previous reports, our method can actually detect IFN-γ in the three samples of aqueous humor from 'acute' uveitis (within 48 h from onset) as well as vast increases of IL-6, IL-8 and MCP-1 (Table 1). It suggested that undetectable levels of IFN-γ in the vitreous samples were not the technical error. Thus, these three factors (IL-6, IL-8 and MCP-1), but not IFN-γ, might be involved in the 'refractory/choronic' uveitis in the VC.

Both IL-6 and IL-23p19 deficiencies reduced Th17 (but intact Th1) responses and ameliorated EAU

Because IL-6 is a pleiotropic inflammatory cytokine and mediates various inflammatory autoimmune diseases [14], we focused on the role of IL-6 in the ocular inflammations. We were also interested in the newly identified Th17, since we and others demonstrated that Th17 was involved in chronic ocular inflammation [30, 31], and IL-6 was recognized as an essential factor in inducing Th17 differentiation from naïve T cells in combination with TGF-β [18]. Moreover, it has been reported that not only IL-6, but also IL-23 is required for optimal Th17 differentiation/expansion [22, 23, 38]. To access the systemic and regional Th17 responses, we used the animal model of human uveitis (EAU).

To investigate the role of IL-6 and Th17 in EAU, we immunized IL-6-deficient, IL-23p19-deficient and control C57BL/6 (B6, WT) mice with IRBP1-20, as described in the Materials and methods section. In all groups, pathological changes in the eye appeared on Day 14, and reached maximal severity on Days 18–22 post-immunization. However, compared with control mice, both IL-6-deficient and, as expected from previous findings [39], IL-23p19-deficient mice showed lower score of uveitis in the entire time course (Fig. 2A and B). Histological examination of the eyes on Day 19 also showed lower cellular infiltrations and/or granuloma formation in IL-6-deficient (Fig. 2C) and IL-23p19-deficient (Fig. 2D) mice than in WT control mice [histological score: 2.2 ± 0.75 (WT) vs 0.88 ± 0.52, $P = 0.0305$, $n = 6$ (IL-6-deficient) and 0.78 ± 0.71, $P = 0.0103$, $n = 9$, (IL-23p19-deficient)].

Next, we assessed IRBP-specific IFN-γ/IL-17 production by T cells from draining lymph node cells. On Day 19, CD4$^+$ T cells from IL-6- and IL-23p19-deficient mice produced significantly less amounts of IRBP-specific IL-17 than those from WT (Fig. 3). IFN-γ levels were not significantly different. These results suggest that IL-6/IL-23p19 deficiency affects only Th17, but not on Th1 responses in EAU model.

Systemic administrations of MR16-1 impaired Th17 responses and ameliorated EAU

To further examine the role of IL-6 in uveitis, we assessed the efficacy of rat anti-mouse IL-6R receptor monoclonal antibody (MR16-1) in EAU by both local (intravitreous) and systemic (intraperitoneally) injection. First we immunized WT mice with IRBP1-20, then injected MR16-1 at a dose of 1 μl per eye of 10 μg/ml into the VC on Day 7 post-immunization. Evaluation of the extent of disease was performed on Day 14. Contrary to our hypothesis, no significant differences were seen between control IgG-injected mice and MR16-1-injected mice (Fig. 4A). It was suggested that regional blockade of IL-6 in the effecter phase led to little effect on developing intraocular inflammation. Another possibility was that 1 μl per eye of 10 μg/ml (i.e. 10 ng per VC) might not be sufficient.

Next, we performed systemic administrations of MR16-1 (10 μg/g body weight) immediately after the immunization as depicted in Fig. 4B, and evaluated EAU on Day 18. This protocol might completely block the effect of IL-6 in the regional lymph node. As shown in Fig. 4D, the fundus presented an obscured margin around the optic disc, retinal exudates and linear vasculitis

TABLE 1. Analysis of cytokine and chemokine in the aqueous humor from acute uveitis patients

Patient	Age (yrs)	Diagnosis	IFN-γ	IL-6	IL-8	MCP-1	G-CSF	GM-CSF
1	36	Behcet's disease	36.3	10 137.7	4046.6	4800.1	<150	660.6
2	64	Unknown	<30	7546.5	357.0	109.8	<150	408.6
2	64	Unknown	437.2	15 675.5	8743.1	2148.6	<150	1480.3
3	54	Unknown	<30	1983.6	235.0	627.9	<150	<150
4	58	FHI	<30	16 504.3	1620.8	5902.7	1018.6	1624.4
5	50	Unknown	<30	63.4	60.3	226.2	<150	<150
6	29	Unknown	<30	70.2	48.1	4307.9	<150	<150
7	35	AAU	85.6	14 556.8	1246.3	1594.6	179.4	1477.3

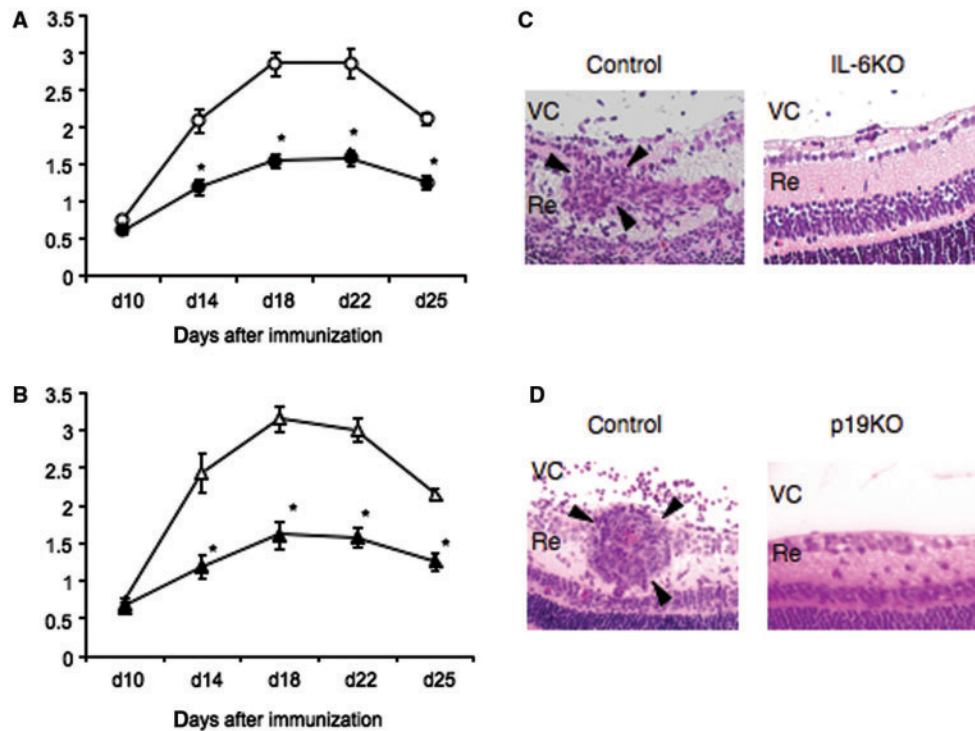All values are given in pg/ml. FHI: Fuchs heterochromic iridocyclitis.

FIG. 2. EAU in IL-6-deficient and IL-23p19-deficient mice. (A) Clinical scores of EAU in IL-6-deficient mice. WT B6 mice (open circles; $n=24$) and IL-6-deficient mice (closed circles; $n=24$) were immunized with IRBP as described in Materials and methods section, and from Day 10, disease severity was examined. The values represent means ± s.e.m. $^*P<0.05$. The data represent combined results of three independent experiments. (B) Clinical scores of EAU in IL-23p19-deficient mice. WT B6 mice (open triangles; $n=14$) and IL-23p19-deficient mice (closed triangles; $n=14$) were immunized as above, and from Day 10, disease severity was examined. The values represent means ± s.e.m. $^*P<0.05$. The data represent combined results of three independent experiments. (C and D) Typical pictures of histological sections in WT B6 mice (left) and IL-6-deficient mice (C, right) and IL-23p19-deficient mice (D, right). The arrows indicate inflammatory cell-infiltration sites.

in the eyes of WT mice. However, in the eyes of MR16-1-treated mice, the margins of optic discs were clear, and only minimal pathological changes, such as retinal vessel dilatation associated with focal vasculitis, were observed (Fig. 4D). Histological examination of the eyes on Day 19 (Fig. 4E) showed lower cellular infiltrations in MR16-1-inoculated mice (histological score: 2.0 ± 0.86 vs 0.88 ± 0.52, $P=0.0188$, $n=8$).

We also assessed the suppressive effect of MR16-1 from the peak of the disease. Although we administered MR16-1 from Day 14 post-immunization at a dosage of 10 and 100 µg/g body weight, disease severity is almost the same (data not shown). Taken together, we concluded that IL-6-mediated inflammation leads to induction of inflammatory cell population and/or macrophage/neutrophil accumulation to the local inflamed site, late administration of MR16-1 could not alter the infiltration of these cells.

To understand the mechanisms of the suppressive effect in MR16-1-treated mice, we examined IRBP-specific IFN-γ/IL-17 production by T cells from draining lymph node cells on Day 19 after immunization. Although CD4$^+$ T cells from MR16-1-treated mice proliferated well in response to IRBP1-20 (data not shown), they produced lower amounts of IL-17 than those from control Ab-treated mice (Fig. 5A). Interestingly, CD4$^+$ T cells from MR16-1-treated mice produced IFN-γ equivalent to control mice. IL-6 has also been showed to play a key role as it inhibits TGF-β-induced regulatory T cell (Treg cells) [18]. One possibility is that the observed reduction of Th17 development and disease progression by MR16-1-treated mice could simply be due to the expansion of Treg cells at the expense of effector cells in an environment that is deficient of IL-6. Foxp3, an X chromosome-encoded forkhead transcription family member, is indispensable for the differentiation of Treg cells [40]. To exclude the possibility described above, we further analysed intracellular Foxp3 expression of MR16-1-treated mice. As shown in Fig. 5B, the population of Foxp3-positive Tregs was not changed significantly in CD4$^+$ T cells from MR16-1-treated mice compared with control IgG-treated mice. These data clearly demonstrated that administration of MR16-1 impaired systemic Th17 response but not Th1 responses, which were similar to either IL-6-deficient mice.
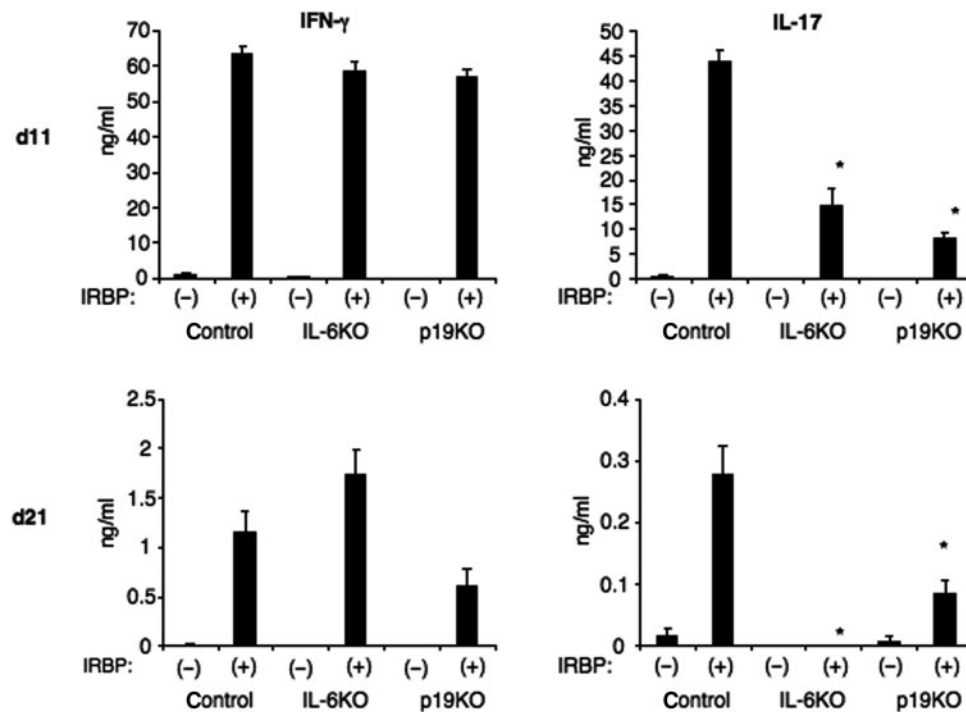
FIG. 3. IRBP-specific cytokine production by LN cells of WT B6, IL-6-deficient and IL-23p19-deficient mice. On Days 11 and 21, LN from WT B6 mice, IL-6-deficient mice and IL-23p19-deficient mice were collected. Purified CD4$^+$ T cells were cultured with 10 μg/ml of IRBP and irradiated (20 Gy) WT APCs. After 48 h, supernatants were analysed for IFN-γ or IL-17 production by ELISA. The values represent means $\pm$ s.d. $^*P < 0.05$.
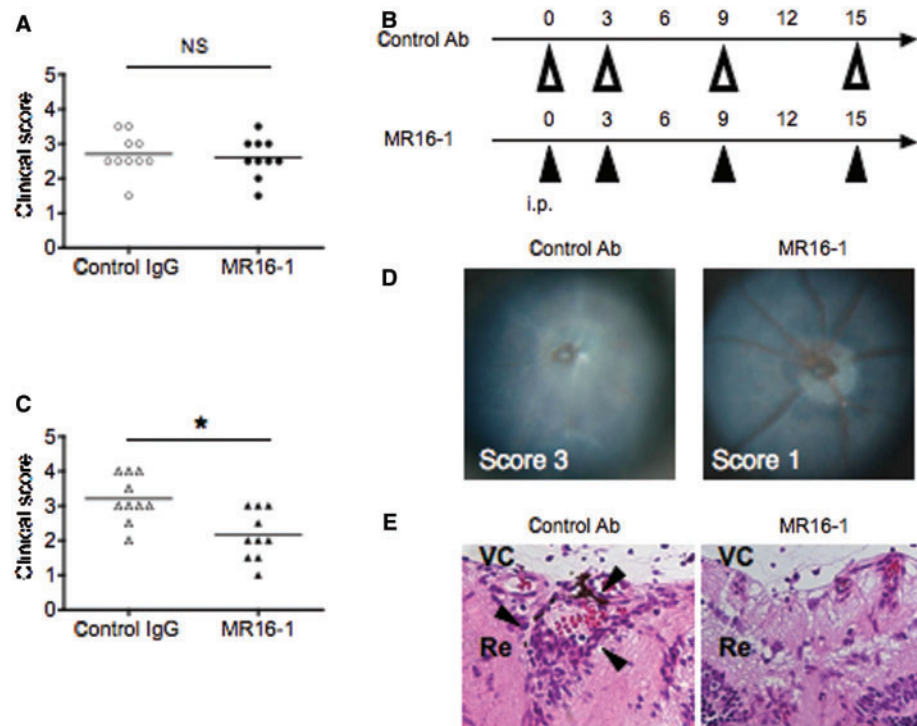

FIG. 4. Effect of administrating IL-6RAb (MR16-1) in EAU. (**A**) Comparison of the funduscopic severity between control Ig-treated (open circles; $n = 10$) and MR16-1-administered (closed circles; $n = 10$) mice. Each antibody was administered intravitreously on Day 7 after IRBP immunization. At Day 14, funduscopic examinations of the mice were performed. (**B**) Intraperitoneal administration protocols of MR16-1 and control Abs. Control IgGs and MR16-1 at the dose of 10 μg/g body weight were administered intraperitoneally four times on days 0, 3, 9 and 15 after IRBP immunization. (**C**) EAU symptoms in the eyes were evaluated 18 days after IRBP immunization. Data shown are control IgGs-treated (open triangles; $n = 13$) and MR16-1-administered (closed triangles; $n = 12$). $^*P < 0.05$ (**D**) Photomicrographs of EAU eye. Typical pictures of ocular fundus of the eye are shown. The EAU scores are indicated in the fundus photographs. (**E**) Photomicrographs of histological sections of the eye. Typical pictures of histological sections in control Ig-administered mice (left) and MR16-1-administered mice (right). The arrows indicate inflammatory cell infiltration sites. Re: retina.
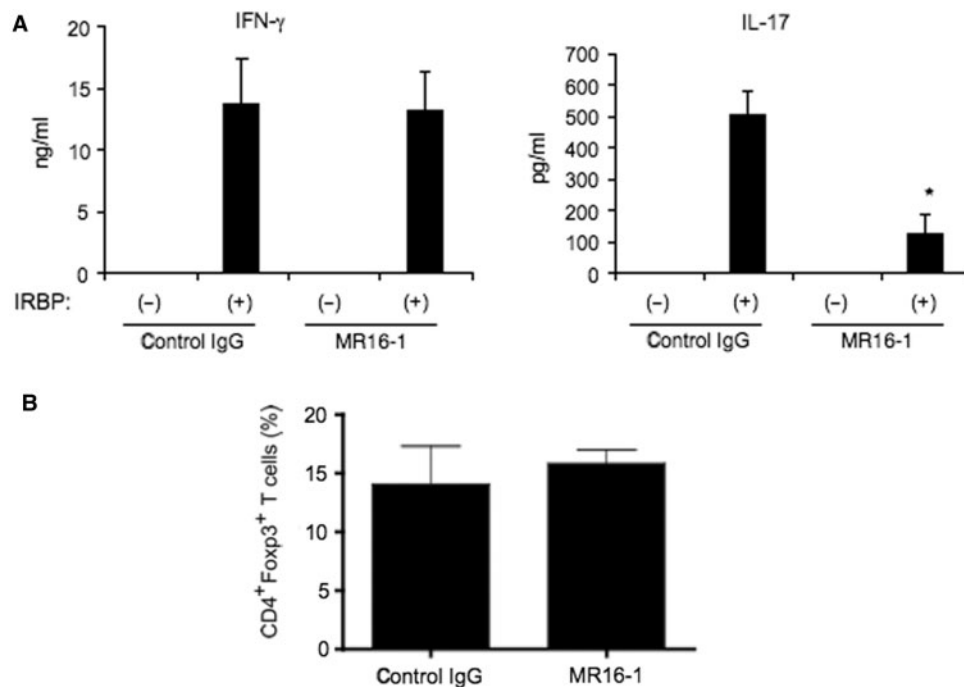
FIG. 5. IRBP-specific cytokine production and Foxp3 expression by LN cells of MR16-1-administered WT B6 mice. On Day 19, LN cells from control IgG-administered mice or MR16-1-administered mice were collected. (**A**) Purified CD4$^+$ T cells were cultured with 10 μg/ml of IRBP and irradiated (20 Gy) WT APCs. After 48 h, supernatants were analysed for IFN-γ or IL-17 production by ELISA. (**B**) Foxp3-positive CD4$^+$ T was analysed by FACS ($n = 7$). The values represent means ± s.d. *$P < 0.05$.

Reduced inflammatory cellular mediators in the eye in MR16-1-treated mice

To investigate the effect of systemic IL-6/Th17 blockade (developing phase) in the local cytokine/chemokine milieu, we measured protein concentrations of the Th1- and/or Th17-related soluble factors in the ocular fluid using the multiplex protein assay (effector phase). We compared MR16-1-treated mice with IL-6-deficient or IL-23p19-deficient mice. On Day 19, IFN-γ and IL-17 concentration in the ocular fluid from all the mutant mice were reduced compared with that from control mice (Fig. 6A and B). Although systemic IL-6/Th17 blockade impaired only Th17 responses in the developing phase, it caused local decrease of cytokines including IFN-γ in the effector phase.

Keino *et al*. [41] demonstrated that Th1-related chemokines, such as IP-10, MCP-1 and regulated on activation, normal T cell expressed and secreted (RANTES), were expressed in the early phase of EAU development. Expression of these chemokines has correlated with the local infiltration of macrophages and neutrophils. In our data, MCP-1 production was significantly reduced in the eyes from IL-6-deficient and IL-23p19-deficient mice, and no change was observed in IP-10 production, albeit they had a tendency to reduce. These data suggested that systemic reduction of Th17 responses results in the decrease of various chemokines, leading to inflammatory cell infiltration.

Discussion

IL-6 as well as IL-23 has been considered to be necessary to generate Th17 [18, 19, 23], which has been described as playing a major role in the pathogenesis of some inflammatory and autoimmune disorders, such as RA [42], chronic obstructive pulmonary disease [43], bone destruction [44], EAE [22] and experimental autoimmune uveoretinitis [30, 31]. In this study, we demonstrated (i) the significant elevation of IL-6 in ocular fluid derived from 'refractory/chronic' uveitis patients, (ii) the contribution of IL-6 and IL-23 as an inducer/expander of antigen-

specific Th17 responses in EAU model and (iii) the therapeutic efficacy of MR16-1 (IL-6R blocking antibody) on EAU via Th17 suppression. Our data suggested IL-6 as well as IL-23 might contribute to ocular inflammatory diseases, through the inflammatory effects by IL-6 and also through their effects on Th17 differentiation/expansion.

We showed three factors (IL-6, IL-8 and MCP-1) involved in the 'refractory/choronic' uveitis in the VC as well as 'acute' uveitis in the aqueous humor, as several investigators previously described [13, 45–48]. Other than IL-6, IL-8 is known to migrate neutrophil infiltration and MCP-1(CCL2) to be monocyte/macrophage infiltration. Both chemokines are now also considered as a new molecular target [49, 50]. In contrast, some major T cell-mediated inflammatory cytokines including IFN-γ, IL-1β, IL-2, IL-4 and IL-10 cannot be detected in 'all' vitreous samples. IFN-γ actually increased aqueous humor of acute uveitis [37], and IFN-γ-producing Th1 cells have been considered as the main cause of EAU [27, 28, 51]. We thus currently consider that IFN-γ mainly affects acute uveitis, but not chronic uveitis. Taken together, we hypothesized that macrophages/neutrophil-dominant inflammation occurs during intraocular inflammation, with little or no T-cell infiltration.

Blocking of IL-6/IL-23 ameliorates EAU in terms of Th17 differentiation/expansion. To investigate the effects of IL-6 as well as Th17, we used three types of experimental line of EAU: blocking IL-6R systemically with MR16-1, IL-6-deficient mice and IL-23p19 deficient mice. All these three experiments displayed amelioration of the disease, and we found that retinal antigen (IRBP)-specific production of IL-17 was significantly reduced from the lymph node cells. Anti-IL-6R antibody (tocilizumab) treatment reported to be effective for several autoimmune diseases: Okada *et al*. [52] reported that blockade of IL-6 receptor suppresses reactive astrogliosis and ameliorates functional recovery in experimental spinal cord injury; Mihara *et al*. [53] reviewed humanized anti-IL-6 receptor antibody as effective for RA, systemic-onset JIA and Crohn's disease. In terms of Th17 suppression, there are several reports demonstrating the
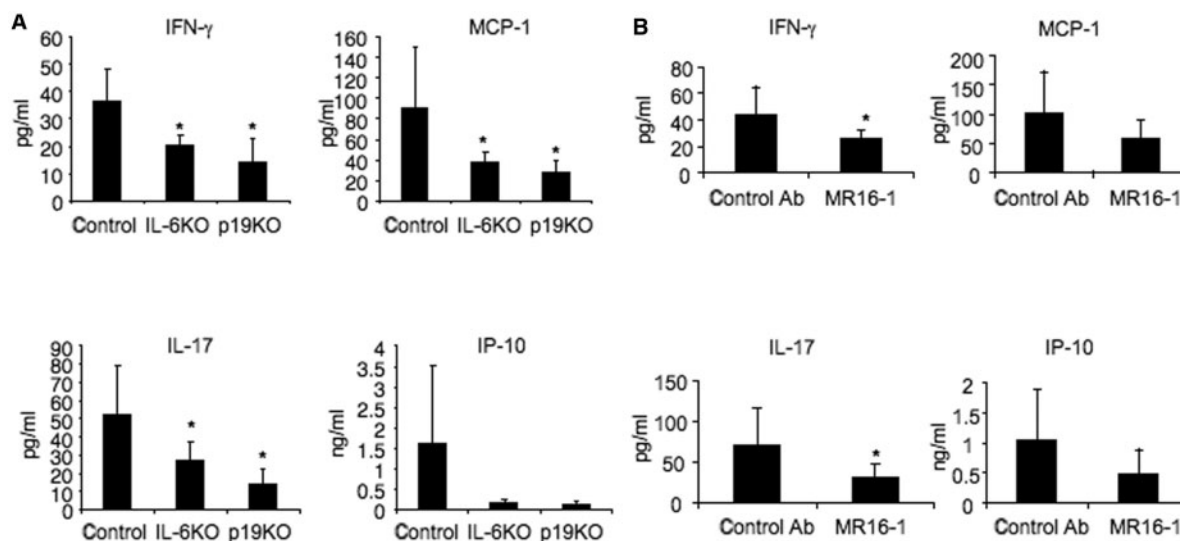
FIG. 6. Cytokines and chemokines in ocular fluid of EAU mice. (**A**) On Day 18, ocular fluid protein from WT B6 mice ($n = 8$), IL-6-deficient mice ($n = 7$) and IL-23p19-deficient mice ($n = 8$) was collected and analysed. (**B**) On Day 19, ocular fluid protein from eyes of control IgG-administered mice ($n = 7$) and MR16-1-administered mice ($n = 7$) was prepared, then the concentrations of indicated cytokine/chemokine production were analysed. The values represent mean $\pm$ s.d. $*P < 0.05$.

contribution of IL-23: Chen *et al.* [24] demonstrated that anti-IL-23 therapy inhibits multiple inflammatory pathways, including Th17 development and ameliorates autoimmune encephalomyelitis; and recently, Luger *et al.* [39] demonstrated that systemic neutralization of IL-23 prevents, but does not reverse, EAU. Taken together, we considered that blocking either IL-6/IL-23 pathway may inhibit Th17 expansion and contributes in suppressing the induction of autoimmune diseases. We noticed that late administration of MR16-1 could not alter the infiltration of inflammatory cells. It was consistent with a previous report [16], and this may be due to the fact that IL-6 plays an important role in the early phase of Th17 differentiation.

Reduced Th17 responses interfere with inflammatory cell infiltration to the retina during EAU. We found that both macrophage and neutrophil infiltration were suppressed in MR16-1-treated, IL-6-deficient and IL-23p19-deficient mice by histological analysis. Several reports provide evidence of the Th17 effect in cellular infiltration; IL-17-producing cells further produce IL-17, IL-6 and TNF-α [22], and these cytokines cause inflammation largely by stimulating fibroblasts, endothelial cells, epithelial cells and macrophages to produce chemokines, as well as G-CSF and GM-CSF, with the subsequent recruitment of PMN [54]. Moreover, Park *et al.* [55] reported that Th17-induced inflammation is dominated by macrophages, with the subsequent production of IL-1, IL-6, MMP3 and inducible nitric oxide syntase. We found that Th17 blockade leads to reduction of chemokines. Therefore, it was suggested that Th17 differentiation/expansion affects both neutrophil and macrophage recruitment into the retina. We thus hypothesize that huge amounts of IL-6 triggers inflammation in circulation, then differentiated Th17 recruits several inflammatory cells to the site (i.e. retina) and infiltrated cells produce more pro-inflammatory cytokines and chemokines, which results in tissue damage. Importantly, existing amounts of IL-6 may also contribute to inflammatory Th17 differentiation.

Our data cannot exclude the possibility that the therapeutic effect of MR16-1 was not mediated solely by Th17 suppression. IL-6 blockade in the developing phase might be able to reduce local IL-8, MCP-1 concentration directly, independent of Th17 responses. IL-6 is produced by variety of cells, i.e. vascular endothelial cells, fibroblasts, keratinocytes and non-specific macrophages, which can further produce inflammatory cytokines and chemokines, and constitute chronic ocular inflammation. Our results from ocular fluids may reflect chronic phases, after

acute Th1 or Th17 inflammation. IL-6 blockade can suppress acute Th17 responses via its differentiation and, importantly, can ameliorate chronic inflammation acts directly on inflammatory cells. Although further studies are necessary, IL-6/IL-23 may be considered a novel target for refractive uveitis.

Rheumatology key messages

- IL-6 is responsible for ocular inflammation.
- Tocilizumab (anti-IL-6R antibody) ameliorates the EAU.

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